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ETS

Prior Exposure to Aged and Diluted Sidestream Cigarette Smoke Impairs Bronchiolar Injury and Repair

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The bronchiolar injury/repair response to naphthalene (NA) in mice includes acute distal airway epithelial injury that is followed by epithelial proliferation and redifferentiation, which result in repair of the epithelium within 14 days. To test whether prior exposure to aged and diluted sidestream cigarette smoke (TS) would alter the injury/repair response of the airway epithelium, adult mice were exposed to either filtered air (FA) or smoke for 5 days before injection with either corn oil carrier (CO) or naphthalene. Mice were killed 1 and 14 days after naphthalene injury. Lung and lobar bronchus were examined and measured using high-resolution epoxyresin sections. The control group (FACOFA) that was exposed to filtered air/corn oil/filtered air contained airway epithelium similar to untreated controls at all airway levels. The group exposed to tobacco smoke/corn oil/filtered air (TSCOFA) contained some rounded cells in the small airways and some expansion of the lateral intercellular space in the larger airways. Necrotic or vacuolated cells were not observed. As expected, the epithelium in the group exposed to filtered air/naphthalene/filtered air (FANAFA) contained many light-staining vacuolated Clara cells and squamated ciliated cells within distal bronchioles during the acute injury phase. Repair (including redifferentiation of epithelial cells and restoration of epithelial thickness) was nearly complete 14 days after injury. The extent of Clara cell injury, as assessed in lobar bronchi, was not different between the four groups. Although the FANAFA group contained greater initial injury in the distal airways at 1 day, the group exposed to tobacco smoke/naphthalene/filtered air (TSNAFA) had the least amount of epithelial repair at 14 days after naphthalene treatment; many terminal bronchioles contained abundant squamated undifferentiated epithelium. We conclude that tobacco smoke exposure prior to injury (1) does not change the target site or target cell type of naphthalene injury, since Clara cells in terminal bronchioles are still selectively injured; (2) results in slightly diminished acute injury from naphthalene in distal bronchioles; and (3) delays bronchiolar epithelial repair.

Key Words: cigarette smoking; tobacco smoke; bronchiolar injury and repair; epithelium; Clara cells; mice.

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Epidemiologic studies have established that involuntary exposure to cigarette smoke at work or in the home has a negative health impact on those who are exposed. Exposure to environmental tobacco smoke (ETS) in the workplace is associated with increased respiratory symptoms including wheezing and cough. The severity of symptoms depends upon the hours per day of smoke exposure (Leuenberger *et al.*, 1994) and may be greater in sensitive populations such as asthmatic patients (Jindal *et al.*, 1994). However, our environment, especially in polluted cities, contains many other pollutants that may also affect respiratory health. What is not known is the impact of ETS exposure on lung injury and repair responses when additional lung injury is initiated by exposure to other toxic pollutants.

One of the regions of the lung particularly prone to injury is the distal conducting airways (bronchioles) at the junction of the terminal bronchiole with the gas exchange region. Due in part to reduction in airway diameter, branching history, and cellular composition, terminal bronchioles are a primary target zone for many oxidant air pollutants, metabolically activated lung toxicants, and toxic particles. In the mouse and other species, the principal bronchiolar cell type, the nonciliated bronchiolar (Clara) cell, contributes to this site selectivity of injury. There are at least two reasons the Clara cell is selectively injured: high abundance within the centriacinar injury target zone, and cellular metabolic characteristics that promote activation of xenobiotics. The Clara cell is the principal site of xenobiotic metabolism within the lung by the cytochrome P450 monooxygenase system (Devereux *et al.*, 1989; Plopper *et al.*, 1987). Clara cell metabolism of xenobiotics by the P450 system, thereby contributing to their own susceptibility, has been further confirmed by metabolite binding within Clara cells (Boyd 1977; Boyd *et al.*, 1980). The injury model we will use exploits the metabolic characteristics of Clara cells to selectively injure them in a well-defined pattern with the bioactivated cytotoxicant naphthalene (Plopper *et al.*, 1992a,b; Van Winkle *et al.*, 1995).

Naphthalene is a ubiquitous environmental pollutant (for review see ATSDR, 1990). It is a major component of cigarette

smoke (Schmeltz *et al.*, 1976; U.S. EPA, 1992) as well as aged and diluted sidestream smoke (Witschi *et al.*, 1997), is present in automobile emissions, and is widely used in the synthesis of insecticides (Life Systems, Inc., 1990; Arey *et al.*, 1987). Susceptibility of mice to naphthalene correlates with formation of the toxic metabolite by cytochrome P450 isozyme 2F (CYP2F), particularly in Clara cells within the injury target zone, distal bronchioles (Buckpitt *et al.*, 1995). Injury is most severe in distal airway regions and increases in a dose-dependent manner up the airway tree (Plopper *et al.*, 1992b). Ciliated cells survive and are not injured regardless of the dose. Mouse Clara cells injured by naphthalene vacuolate and then exfoliate 1–2 days after injury by a 200-mg/kg dose of naphthalene (Van Winkle *et al.*, 1995). In this study we ask the question whether a history of prior exposure to ETS modulates the pattern of naphthalene-induced distal conducting airway Clara cell injury and subsequent repair.

There are many human lung diseases (including cancer, bronchitis, bronchiolitis, and asthma) that appear to be the result of abnormal repair. The hypothesis we are testing is that exposure of nonsmokers to environmental tobacco smoke predisposes their lungs to injury by inhaled and ingested pollutants and that smoke exposure compromises the ability of the lung to repair following acute injury. The long-term sequela may be airway remodeling. It is not known if cigarette smoke exposure before bronchiolar injury results in a site-specific bronchiolar lesion similar to that without prior ETS exposure, increases or decreases the susceptibility of Clara cells to additional cytotoxicants, or alters the repair capability of bronchiolar epithelium. To address these questions we used a combination of histologic and morphometric approaches based on detection of site-specific responses using airway microdissection to isolate specific regions of the lung. We found that a week of prior exposure to tobacco smoke, using a regimen that mimics an occupational exposure 5 days/week, 6 h/day, resulted in prolonged bronchiolar repair from naphthalene injury in terminal bronchioles.

MATERIALS AND METHODS

Animals. Adult (8-week-old) male Swiss Webster mice (CFW, Charles River) were housed in AAALAC-approved facilities on a 12/12 light/dark cycle with food and water *ad libitum*. All animals were treated with naphthalene or corn oil at the same time of day, between 8 and 10 A.M. Animals were killed at 1 and 14 days after treatment with either 200 mg/kg naphthalene or a corresponding volume of vehicle (corn oil). The number of animals in each group was four. The trachea was cannulated and lungs were removed from the chest. The left lobe was removed and cannulated separately for fixation with 1% paraformaldehyde at 30 cm of pressure for 1 h, paraffin processed, and sectioned for use with immunohistochemical techniques. The right half of the lung was inflated to capacity with 37°C 5 μM ethidium homodimer-1 (Molecular Probes, Eugene OR) in Ham's F12 media for 10 min and then lavaged three times with 37°C F12 media as previously described (Van Winkle *et al.*, 1999). The right lobes were fixed at 30 cm of pressure with 330 mOsm Karnovsky's fixative (glutaraldehyde/paraformaldehyde) for 1 h. Lungs were stored in fixative in the dark until used.

TABLE 1
Average Daily Data for ADSS Exposure

	Average ± SD
Temperature (°F)	72.63 ± 1.36
Humidity (% RH)	56.56 ± 7.02
Carbon monoxide (ppm)	5.69 ± 0.83
Nicotine (μg/m^3)	135.69 ± 82.42
Piezobalance TSP (mg/m^3)	1.00 ± 0.05

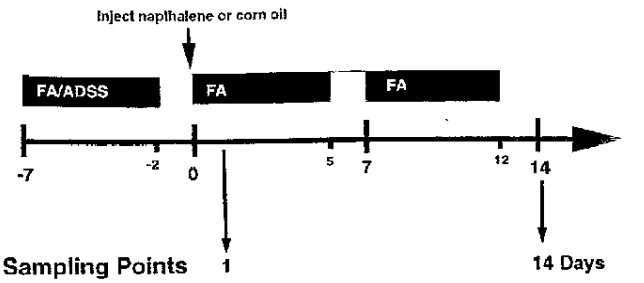

FIG. 1. Timeline that corresponds to treatment protocol and sampling points in the experimental design. Numbers represent days. Adult male mice were pretreated for 5 days with either filtered air (FA) or aged and diluted sidestream cigarette smoke (ADSS), continued for 2 days in filtered air, and treated between 8 and 10 AM with either naphthalene or corn oil (carrier) by ip injection (Day 0). The mice then continued in FA for up to 14 days. Mice from various treatment groups (four mice/group) were killed on Days 1 and 14 after naphthalene or corn oil injection.

Exposure protocol. Mice were exposed 5 days/week, 6 h/day to approximately 1 mg/m^3 total suspended particulates (TSP) of aged and diluted sidestream smoke (ADSS) as a surrogate for ETS (for exposure conditions see Table 1). The remaining 2 days/week, mice were not exposed to ADSS and received only filtered air. Both filtered air (sham)-exposed and smoke-exposed mice were housed continuously in plastic shoebox cages in updated Hinners chambers. The exposure system and monitoring methods have been previously described in detail (Joad *et al.*, 1995; Teague *et al.*, 1994). The ADSS is generated by a modified ADL/II smoke exposure system (Oakridge National Laboratory) using conditioned cigarettes from the Tobacco and Health Research Institute of the University of Kentucky. Two cigarettes at a time were smoked under Federal Trade Commission conditions in a staggered fashion at a rate of 1 puff (35 ml, 2-s duration)/min. The sidestream smoke was drawn into a conditioning chamber where it was aged and diluted. The sidestream smoke was further diluted as it was passed into the exposure chambers in such a way as to produce total suspended particulate (TSP) concentrations of 1.0 mg/m^3. Relative humidity, temperature, TSP, and nicotine concentrations were sampled daily over the 3-week exposure time period (Table 1). After 1 week of exposure to either filtered air (FA) or ADSS, the mice were injected intraperitoneally (ip) with either corn oil carrier or 200 mg/kg NA and returned to either ADSS or FA for 1 or 14 days. See timeline of exposure (Fig. 1) and the list of the experimental groups (Table 2) for further details of the experimental design.

High-resolution light microscopy of various airway levels. The right cranial lobe of Karnovsky's fixed lungs was sliced (approximately 3 mm thick) perpendicular to the axial pathway of the major daughter bronchus in such a way as to bracket the first two minor daughter airway branches immediately distal to the hilum. Only terminal bronchioles from the same two minor daughter branches were evaluated for each animal, to minimize the possible

TABLE 2
Acute Adult (8 Weeks Old) Mouse Exposure to ADSS. Total Exposure: 1 Week

Group	*n*/time point	*n*/exposure group	Sampling points
FACOFA	4	8	1 day and 14 days
TSCOFA	4	8	1 day and 14 days
FANAFA	4	8	1 day and 14 days
TSNAFA	4	8	1 day and 14 days

Note. TS, smoke exposed; FA, filtered air exposed; CO, corn oil (carrier) treated; NA, naphthalene treated. All exposures/treatments are listed in chronological order to define each group. Days, number of days after naphthalene or corn oil injection. Mice were treated with naphthalene at the beginning of Week 2.

effect of the differential deposition of tobacco smoke with different airway branching patterns on the results. Each branch was dissected to the last three to six distal branching generations of bronchioles prior to the axial terminal bronchiole and embedded. A cross section of the trachea and a cross section of the lobar bronchus were also dissected free of surrounding tissue. These samples were postfixed in 1% osmium tetroxide in Zetterquist's buffer, processed by large-block methodology, and embedded in Araldite 502 epoxy resin. Specimens were sectioned at 1 μm on a Sorvall JB4 microtome with glass knives, stained with methylene blue/Azure II, and imaged on an Olympus Provis microscope.

Quantitative histopathology. The abundance of normal and cytotoxic bronchiolar epithelial cells was analyzed using morphometric procedures previously used to define changes in bronchiolar epithelium after naphthalene injury (Plopper *et al.*, 1992a) and discussed in detail by Hyde *et al.* (1990). Only terminal bronchioles from the same two minor daughter airways were evaluated for each animal, to minimize the possible effect of the differential deposition of tobacco smoke on the results. All terminal bronchioles visible on sections from two separate block faces from each animal (five terminal bronchioles was the minimum number obtained) were evaluated. All the measurements were made using high-resolution 1.0-μm sections. The volume densities (V_v) of five categories of cells (nonciliated Clara, ciliated, vacuolated, squamous, and other) were defined by point (P) and intercept (I) counting of terminal bronchiolar epithelial vertical profiles using a cycloid grid and Stereology Toolbox (Morphometrix) on images collected as described earlier. V_v was calculated using the formula

$$V_v = P_p = P_n/P_t$$

where P_p is the point fraction of P_n, the number of test points hitting the structure of interest, divided by P_t, the total points hitting the reference space (epithelium). The surface area of epithelial basement membrane per reference volume (S_v) is determined by point and intercept counting and calculated using the formula

$$S_v = 2\ I_e/L_t$$

where I_e is the number of intersections with the object (epithelial basal lamina) and L_t is the length of the test line in the reference volume (epithelium). The thickness of the epithelium, or volume per unit area (V_v) of basal lamina ($\mu m^3/\mu m^2$), was calculated using the formula for arithmetic mean thickness (t)

$$t = V_v/S_v$$

Immunohistochemistry. The presence of the differentiation marker proteins was detected using specific antibodies: rabbit anti-rat CC10 and rabbit anti-mouse CYP2F2 (Nagata *et al.*, 1990; Singh and Katyal, 1984). The avidin-biotin peroxidase procedure was used to identify antibody binding sites. The procedure was the same as outlined by the supplier (Vector Labs, Burlingame, CA). Diaminobenzidine with nickel enhancement was used as the chromagen. To eliminate nonspecific binding of the primary antibody, sections were blocked with 1% bovine serum albumin. Controls included substitution of phosphate-buffered saline (PBS) for the primary antibody. The optimal dilution at which there was positive staining with minimal background staining was determined separately for each antibody using a series of dilutions on sections from corn oil- and naphthalene-treated animals. Rabbit antisera specific for rat CC10 was a generous gift from Dr. Gurmukh Singh (Veterans Affairs Medical Center, Pittsburgh, PA). Rabbit antisera specific for mouse CYP2F2 was provided by Dr. Henry Sasame (National Institutes of Health, Bethesda, MD).

Scanning electron microscopy (SEM). Samples were microdissected from the main axial path of the cardiac lobe so that distal bronchioles, including terminal bronchioles, and distal and proximal intrapulmonary bronchi were exposed. Samples for SEM were dehydrated in a graded ethanol series, rinsed in a graded ethanol toluene series, returned to ethanol immersed in hexamethyldisilizane (Polysciences, Inc., Warrington, PA) for 5 min, then air dried overnight at room temperature, as described previously (Nation, 1983). Samples were mounted on carbon-coated stubs and coated with gold in a sputter-coater (Polaron II E5100) with 2.5 kV acceleration voltage in an argon atmosphere with a current of 10 mA for 2 min. Samples were imaged with a Phillips SEM 501 at 500 and 1250 magnification (Malwah, NJ).

Statistical analysis. Morphometric data from all terminal bronchioles present in two block faces, a minimum of five terminal bronchiolar epithelial profiles, were used to calculate V_v or V_s per animal. The value per animal was used to calculate the mean and standard deviation for each group of animals at a time point. Four animals were measured at each time point. Because the data failed the usual ANOVA assumptions regarding homogeneity of variance, data were analyzed by use of an ANOVA weighted by the reciprocal of the "in treatment" variance (Neter *et al.*, 1990). The significance of post hoc comparisons was determined using Tukey's HSD method at the 0.05 level (Neter *et al.*, 1990).

Reagents. Naphthalene was purchased from Fisher Scientific (Pittsburgh, PA). Corn oil (Mazola) was manufactured by Best Foods/CPC International Inc. (Englewood Cliffs, NJ). Glutaraldehyde, paraformaldehyde, Azure II, and Araldite 502 were obtained from Electron Microscopy Sciences (Fort Washington, PA). Osmium tetroxide was purchased from Polysciences, Inc. (Warrington, PA). Methylene blue was from JT Baker Chemical (Phillipsburg, NJ).

RESULTS

Histopathology

The terminal bronchiolar epithelium in mice in the sham-treated group (FACOFA) consisted of cuboidal cells that were predominantly either nonciliated bronchiolar (Clara) or ciliated cells at 1 day after corn oil injection (Figs. 2A and 2B) and at 14 days after corn oil injection (Figs. 3A and 3B). In mice exposed to tobacco smoke and sham injected (TSCOFA), the epithelial cells in the terminal bronchioles (Figs. 2C and 2D) had a more rounded profile than epithelial cells in control animals. Even after 2 additional weeks of filtered air exposure, when the majority of the terminal bronchiolar epithelial cells had regained a more uniform appearance, a few cells still appeared swollen (Fig. 3D). In mice exposed to naphthalene only (FANAFA), the acute injury phase was evident 1 day after naphthalene injection. Vacuolated cells were located in the

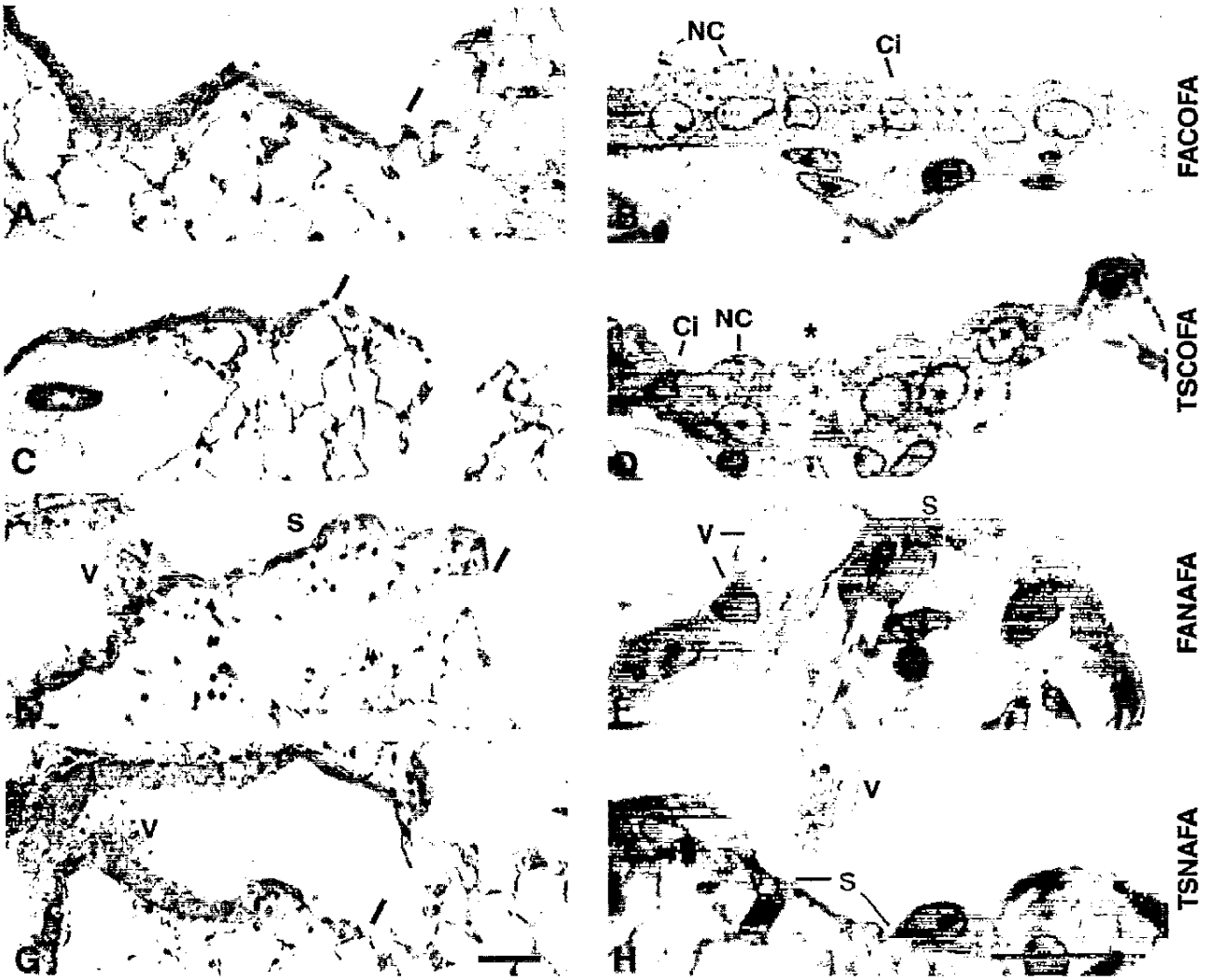

FIG. 2. High-resolution histopathology of terminal bronchioles (TB) of mice with and without a 5-day prior exposure to tobacco smoke (TS) at 1 day after injection with either naphthalene or corn oil. The junction of the terminal bronchiole with the alveolar duct is noted by a line in the left column of micrographs. (A and B) Cuboidal bronchiolar epithelium in a sham-treated control animal (FACOFA) includes both nonciliated Clara cells (NC) and ciliated cells (Ci). (C and D) The epithelium of an animal previously exposed to tobacco smoke for 5 days in filtered air (TSCOFA) contains some rounded light-staining cells (*) as well as Clara cells (NC) and ciliated cells (Ci). (E and F) The acute epithelial response after naphthalene injury is characterized by vacuolation and exfoliation of nonciliated Clara cells. Note the vacuolated epithelial cell (V) in the animal exposed to naphthalene only. (G and H) In mice previously exposed to tobacco smoke and then treated with naphthalene (TSNAFA), vacuolated and exfoliated Clara cells are present (V). The basement membrane is covered by squamated ciliated cells (S). Low magnification (left column) is included to show the extent of epithelial changes in the terminal bronchiole. Note the larger extent of epithelial vacuolation, exfoliation, and squamation (S) in the terminal bronchioles of mice exposed to naphthalene only (E and F) compared to those with only prior smoke exposure (C and D) or those with both prior smoke exposure and naphthalene treatment (G and H). Bar in (G) = 50 μm; bar in (H) = 20 μm.

airway epithelium as well as exfoliated into the lumen of the distal airways (Figs. 2E and 2F). Squamated cells were also present (Fig. 2F). Fourteen days after naphthalene injection, the epithelium in the FANAFA group once again consisted of predominantly cuboidal epithelial cells (Figs. 3E and 3F). The terminal bronchiolar epithelium of all naphthalene-treated animals was similar 1 day after naphthalene injection (compare Figs. 2E–2H), with both squamated ciliated cells and vacuolated Clara cells present. There was some variability between bronchioles in the same mouse. However, the general injury pattern, which included selective injury of distal airway Clara cells, was similar in the groups treated with naphthalene (FANAFA, TSNAFA). The extent of initial injury within the terminal bronchiole appeared slightly greater in the group that received naphthalene only, FANAFA, compared with the group that was exposed to smoke and then treated with naph-

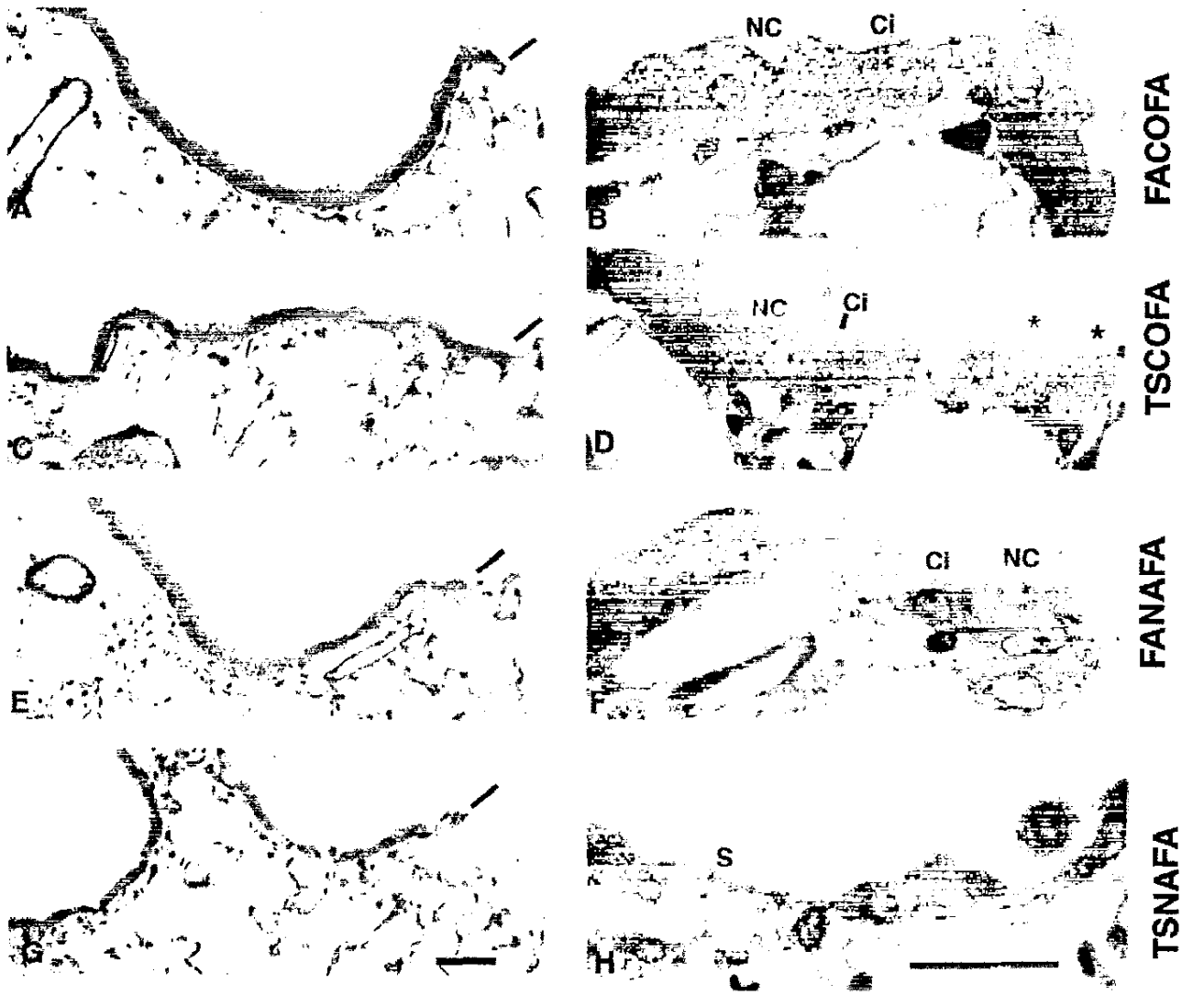

FIG. 3. High-resolution histopathology of terminal bronchioles (TB) of mice with and without a 5-day prior exposure to tobacco smoke (TS) at 14 days after either naphthalene or corn oil injection. The junction of the terminal bronchiole with the alveolar duct is noted by a line in the left column of micrographs. (A and B) Normal cuboidal epithelium in a sham-treated control animal (FACOFA) includes both nonciliated Clara cells (NC) and ciliated cells (Ci). (C and D) The epithelium of an animal exposed to tobacco smoke for 5 days followed by 17 days in filtered air (TSCOFA) still contains some rounded cells (*) as well as Clara cells (NC) and ciliated cells (Ci). (E and F) Epithelial repair after naphthalene injury is characterized by near return to steady state by 14 days after naphthalene treatment. Note the cuboidal epithelium of somewhat uneven thickness in the animal exposed to naphthalene only (FANAFA) that contains both Clara cells and ciliated cells. (G and H) In mice previously exposed to tobacco smoke and then treated with naphthalene (TSNAFA), the basement membrane is still covered in some areas by squamated cells (S). The epithelial thickness is remarkably variable. Low magnification (left column) is included to show the extent of epithelial changes in the terminal bronchiole. Note the extensive epithelial squamation in the terminal bronchioles of mice exposed to tobacco smoke and naphthalene (G; TSNAFA). Bar in (G) = 50 μm; bar in (H) = 20 μm.

thalene, TSNAFA (compare Figs. 2E and 2F with Figs. 2G and 2H), in that fewer vacuolated and more squamated cells were apparent at the most distal ends of the terminal bronchioles. The repair pattern in the group of animals exposed to both naphthalene and tobacco smoke in combination, TSNAFA, differed from animals that were exposed to naphthalene alone, FANAFA, in that abundant squamous epithelium was still present in the terminal bronchioles 14 days after naphthalene treatment (compare Figs. 3G and 3H with Figs. 3E and 3F).

The extent of the initial naphthalene injury was further examined using high-resolution light microscopy on sections of large airways, lobar bronchus, from mice 1 day after injection with either naphthalene or corn oil (Fig. 4). All treatment groups contained cuboidal epithelium consisting of ciliated,

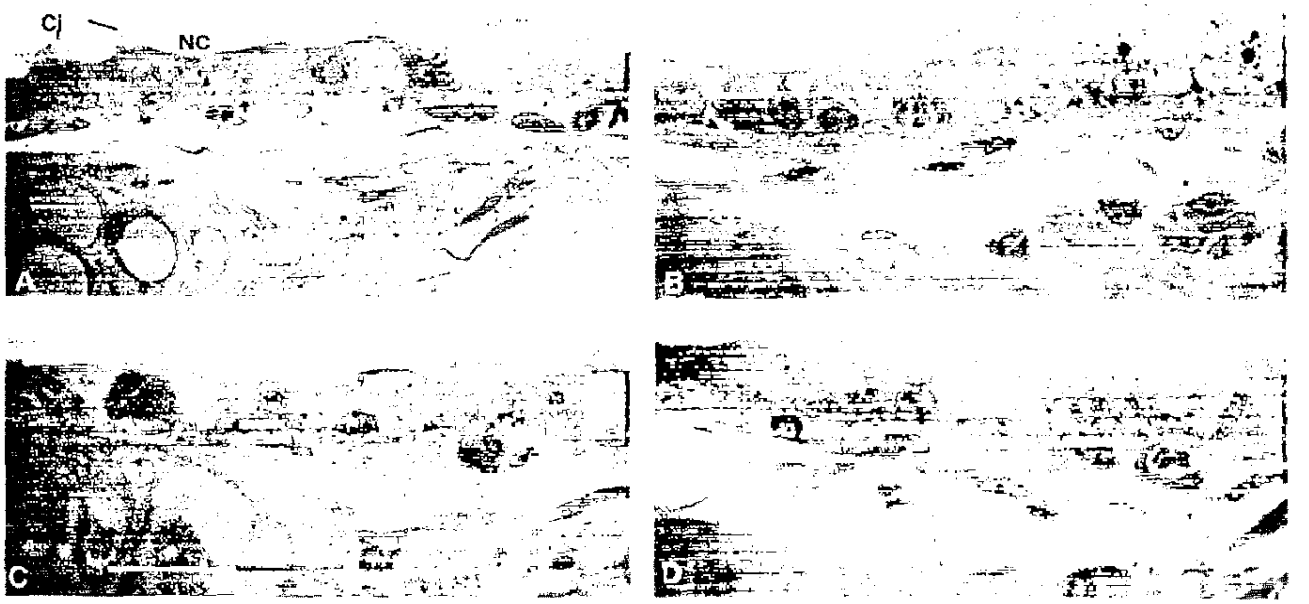

FIG. 4. High-resolution histopathology of the lobar bronchus of mice with and without a 5-day prior exposure to tobacco smoke (TS) at 1 day after naphthalene or corn oil injection. (A) Bronchial epithelium of a sham-treated control animal (FACOFA) contains Ci cells and NC cells. (B) In the bronchial epithelium of an animal previously exposed to tobacco smoke for 5 days (TSCOFA), a slight enlargement of the lateral intercellular spaces is evident (arrows), but there is no frank cytotoxicity to either ciliated or nonciliated cells. (C and D) Both groups of mice exposed to naphthalene with (D; TSNAFA) and without (C; FANAFA) prior smoke exposure contained normal-appearing ciliated and nonciliated epithelial cells. Bar = 20 μm.

Clara, and basal cells (Figs. 4A–4D). However, some cells did contain clear cytoplasmic inclusions (Figs. 4A–4D), and these cells were found in all groups.

Terminal bronchiolar epithelial cellular redifferentiation during repair was further evaluated using immunohistochemistry of Clara cell differentiation markers (CC10 and CYP2F) as well as SEM. SEM was performed on cardiac lobes that had been microdissected to expose the last conducting airway generations, the terminal bronchioles (Fig. 5E). A comprehensive low-magnification map of the distal airway surface was generated in this manner. The terminal bronchioles of the main axial airway path are shown in Figures 5A–5D for the four treatment groups. The epithelium in the FACOFA, TSCOFA, and FANAFA groups (Figs. 5A–5C) consisted predominantly of Clara cells with their characteristic protruding apex and ciliated cells. Some small patches of cells (Fig. 5C, arrowhead) that lacked either apical protrusions or cilia were found in all three groups. Small patches of undifferentiated cells were more frequently noted in the FANAFA group than in the FACOFA and TSCOFA groups. However, the FANAFA group also had terminal bronchioles that lacked squamated cell patches. In contrast, the entire terminal bronchiolar epithelium in the TSNAFA group had a more flattened appearance (Fig. 5D). Although ciliated cells were present (Fig. 5D, inset) the typical protruding apex of differentiated Clara cells were largely absent. When Clara cell differentiation marker proteins CC10 (Figs. 6A, 6C, and 6E) and CYP2F (Figs. 6B, 6D, and 6F) were detected using immunohistochemistry on paraffin sections, the terminal bronchiolar epithelium of the nearly repaired FANAFA group (Figs. 6A and 6B) contained numerous Clara cells that expressed abundant amounts of both proteins. The distribution and abundance of CC10 and CYP2F were similar in the FANAFA and FACOFA groups at 14 days (not shown). However, the group of animals exposed to smoke and naphthalene (TSNAFA) contained fewer differentiated Clara cells; both proteins were found in fewer cells and at decreased abundance compared to the group that received naphthalene only (compare Figs. 6C and 6D with Figs. 6A and 6B). When PBS was substituted for the primary antibody on sections from sham-treated controls (FACOFA), no staining was detected (Figs. 6E and 6F).

Morphometry

High-resolution epoxy resin sections for all four treatment groups were analyzed 14 days after injection of the animals with either naphthalene or corn oil. All cell types were defined based on their morphologic appearance at high magnification. Nonciliated bronchiolar (Clara) cells were distinguished by their protruding apex and cuboidal shape as well as the presence of granules. Ciliated cells were defined by their cuboidal shape and the presence of cilia as well as a darker-staining cytoplasm with a basally located nucleus. Squamated cells were defined as cells that lacked either cilia or Clara cell

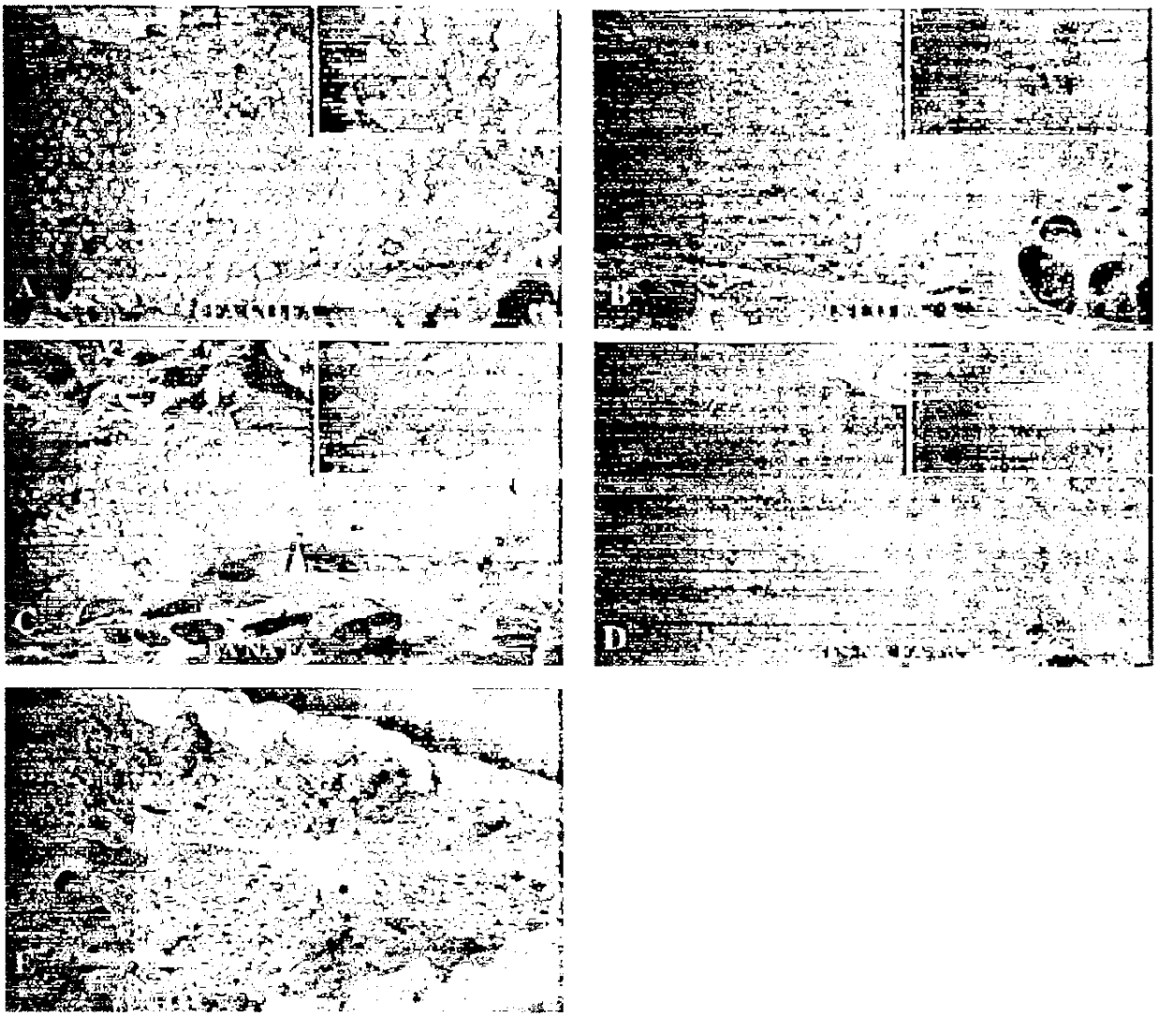

FIG. 5. Scanning electron micrographs of terminal bronchiolar epithelium from mice 14 days after treatment with either corn oil (CO) or naphthalene (NA). Groups were also coexposed to either tobacco smoke (TS) or filtered air (FA) for 5 days prior to naphthalene injury. (A) Clara cells were readily identified by their protruding apices and ciliated cells were clearly defined by their cilia in the sham-exposed animals (FACOFA). (B) Clara cell apical protrusions were more blunted in the animals exposed to tobacco smoke for 5 days followed by 17 days in filtered air (TSCOFA). (C) A single patch of undifferentiated squamous cells (arrowhead) is present in the terminal bronchiole of a mouse that was injured with naphthalene only (FANAFA). This terminal bronchiole is nearing completion of the post-injury repair phase. (D) In contrast, the terminal bronchioles of mice exposed to tobacco smoke before injury with naphthalene have not repaired (TSNAFA). The epithelium contains many squamated and undifferentiated cells that lack either cilia or protruding Clara cell apices. (E) Fixed lung lobes were microdissected along the main axial airway path to the last conducting airway generations, the terminal bronchioles. This allowed the lumenal morphology of all the cells lining many terminal bronchioles to be observed. Bar in (E) = 50 μm; bar in (E) inset = 20 μm; bar in (F) = 500 μm.

granules and in which the base of the cell was more than four times the height. In addition, cells were also classified as "vacuolated" or "other". Vacuolated cells were generally Clara-like cells with areas of large clear cytoplasmic spaces. A cell was considered to be vacuolated if any part of it contained these spaces. Cells that could not be defined were counted in the "other" category. Generally these cells were low cuboidal and lacked characteristics of either Clara cells or ciliated cells. Clusters of cells that resembled neuroepithelial bodies were included in the "other" category and were infrequent in the terminal bronchiolar epithelium.

Epithelial thickness (t), or the total volume (μm^3) of all the epithelial cells per area of basal lamina surface (μm^2), was measured for all four treatment groups and analyzed using a weighted ANOVA for treatment effect, which was significant at $p \leq 0.0095$ (Fig. 7). Sham-treated controls, FACOFA, had

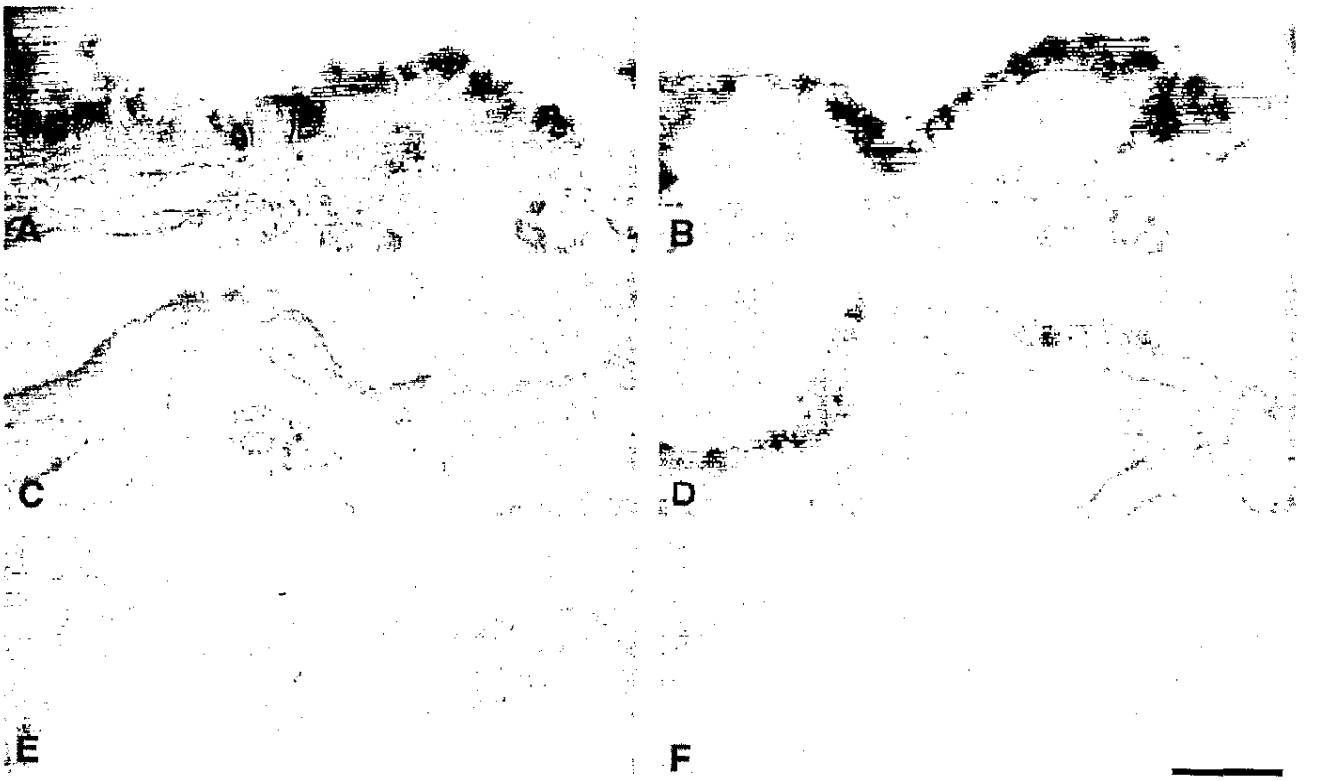

FIG. 6. Terminal bronchiolar epithelium from mice 14 days after treatment with naphthalene (NA). (A, C, and E) Immunohistochemical detection of the Clara cell differentiation marker for Clara cell secretory protein CC10. (B, D, and F) Immunohistochemical detection of the Clara cell differentiation marker for cytochrome P450 isozyme 2F2. (A and B) Both CC10 and CYP2F are found in high abundance in the redifferentiated Clara cells in the nearly repaired bronchioles of mice treated with naphthalene (FANAFA). (C and D) In contrast, the epithelium of mice exposed to tobacco smoke before treatment with naphthalene is considerably less differentiated (TSNAFA). (E and F) When PBS was substituted for the primary antibody on sections from sham-treated control mice (FACOFA), there was no staining. Bar = 20 μm.

the highest mean value (7.86 ± 0.76 $\mu m^3/\mu m^2$). The TSCOFA and FANAFA groups had lower mean epithelial thickness (6.56 ± 0.98 $\mu m^3/\mu m^2$ and 6.78 ± 0.50 $\mu m^3/\mu m^2$, respectively) but were not significantly different from sham-treated controls. In contrast, epithelial thickness was significantly reduced (5.42 ± 0.86 $\mu m^3/\mu m^2$) in the TSNAFA group compared to controls. The volume per surface (V_s) of the three main epithelial cell types is shown in Figure 8. There was a significant treatment effect for both the V_s of nonciliated cells ($p \leq 0.0001$) and the V_s of squamous cells ($p \leq 0.0004$). However, the treatment effect was found to be insignificant for V_s of ciliated cells. When groups were compared against each other using Tukey's post hoc comparison test, V_s of nonciliated cells was significantly different ($p < 0.05$) for all treated groups (TSCOFA, FANAFA, TSNAFA) compared to controls. In addition, the V_s of nonciliated cells for the TSNAFA group was significantly different from the group that received naphthalene only, FANAFA ($p < 0.05$). The V_s of squamous cells was only significantly different from sham-treated controls (FACOFA) for the two groups that received naphthalene (FANAFA and TSNAFA). When each of the cell types was expressed as the volume fraction of total epithelial volume (V_v, Table 3), only the fractions for nonciliated, squamous, and "other" cell classifications had a significant treatment effect by ANOVA. Again, the two groups treated with naphthalene (FANAFA and TSNAFA) were significantly different from controls for V_v nonciliated, V_v squamous, and V_v "other" cell classifications. In addition, for V_v nonciliated, the group exposed to tobacco smoke only (TSCOFA) was also significantly different from controls (FACOFA) and the group that received both naphthalene and smoke (TSNAFA).

DISCUSSION

The goal of this study was to establish whether a short-term (1 week) occupational exposure to environmental tobacco smoke alters acute airway injury or subsequent bronchiolar epithelial repair. Bioactivated Clara cell cytotoxicants, such as aromatic hydrocarbons like naphthalene, are common environmental pollutants to which smokers are likely to be coexposed by either inhalation or ingestion. Naphthalene is used in this study as a surrogate for bioactivated toxicants that affect Clara

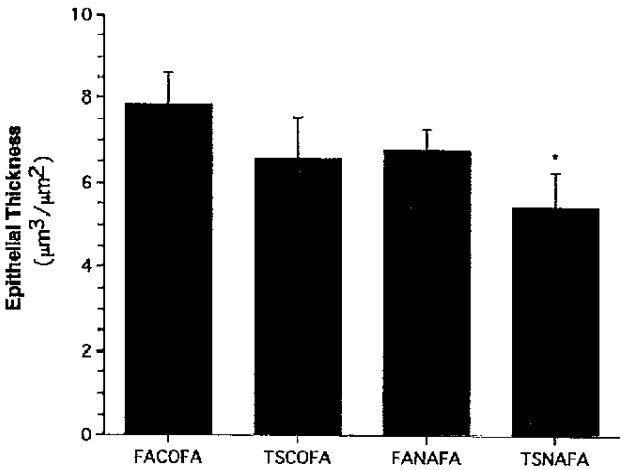

FIG. 7. Epithelial thickness (t) in terminal bronchioles of mice from the seven treatment groups. Three groups (FACOFA, TSCOFA, FANAFA) did not differ significantly from each other by weighted ANOVA and Tukey's post hoc comparison test. However, the mice (TSNAFA) that were exposed first to tobacco smoke (TS) and then treated with naphthalene (NA) had epithelial thickness in their terminal bronchioles that was significantly reduced compared to sham-treated controls (FACOFA). *Significantly different from FACOFA at $p < 0.05$.

cells because its injury and repair pattern have been well characterized for this sex and strain of mouse (Plopper *et al.*, 1992b; Van Winkle *et al.*, 1995). Acute Clara cell injury and repair is a multiphase process that includes the overlapping phases of bronchiolar Clara cell vacuolation and exfoliation, squamation of the surviving ciliated cells to cover the basal lamina, proliferation, migration, and eventual redifferentiation of surviving cells and regeneration of the steady state cuboidal bronchiolar epithelium consisting of Clara cells and ciliated cells (Stripp *et al.*, 1995; Van Winkle *et al.*, 1995, 1997). At the dose used in this study, naphthalene injures Clara cells only in the distal 2–3 airway generations of bronchioles before the gas exchange region (Plopper *et al.*, 1992b; Van Winkle *et al.*, 1995). This pattern of injury was recapitulated in this study whether or not a prior exposure to tobacco smoke occurred: only Clara cells in the most distal airways were injured. However, Clara cells in the distal bronchioles from mice previously exposed to tobacco smoke were slightly less affected by naphthalene injury. Both groups treated with naphthalene underwent the same sequence of acute injury events: exfoliation of Clara cells was accompanied by squamation of the remaining ciliated cells to form a very thin layer of undifferentiated cells (lacking Clara cell apical protrusions or cilia) covering the basal lamina in terminal bronchioles, as has been previously described (Van Winkle *et al.*, 1995). Although the acute injury patterns at 1 day after injection were similar, the repair pattern differed markedly between the two groups that were injured with naphthalene. The repair process was delayed or inhibited in mice that were preexposed to tobacco smoke before naphthalene treatment. The terminal bronchiolar epithelium did not return to steady state, but contained abundant undifferentiated squamated cells 14 days after naphthalene injury. In contrast, mice exposed only to naphthalene underwent substantial epithelial regeneration to return the terminal bronchiolar epithelium to near steady-state levels of epithelial differentiation and density by 14 days after injury. This suggests that prior ETS exposure delays or inhibits bronchiolar epithelial repair and Clara cell redifferentiation after acute Clara cell injury by bioactivated cytotoxicants.

As naphthalene injury increases in severity with increasing dose, it affects more proximal airway generations (Plopper *et al.*, 1992b). One goal of this study was to determine if prior ETS exposure would increase the severity of acute injury (as indicated by histopathology at 1 day after naphthalene injury) to include large airways such as lobar bronchi that are not normally injured by this dose of naphthalene in this strain and sex of mouse. The extent of naphthalene injury was not expanded to lobar bronchi by prior exposure to ETS. However, within the injury target zone, terminal bronchioles, injury due to naphthalene was slightly attenuated in mice that had been previously exposed to tobacco smoke. The injury was slightly less extensive; fewer Clara cells were vacuolated and exfoliated than in the terminal bronchioles from the group treated with naphthalene only (compare Figs. 2F and 2G). It should be emphasized that this effect was slight and was only present in the most distal airways. The reduced Clara cell injury found in this site may reflect the conditioning effect of prior exposure to naphthalene in ETS. ADSS generated at 78 mg/m^3 TSP con-

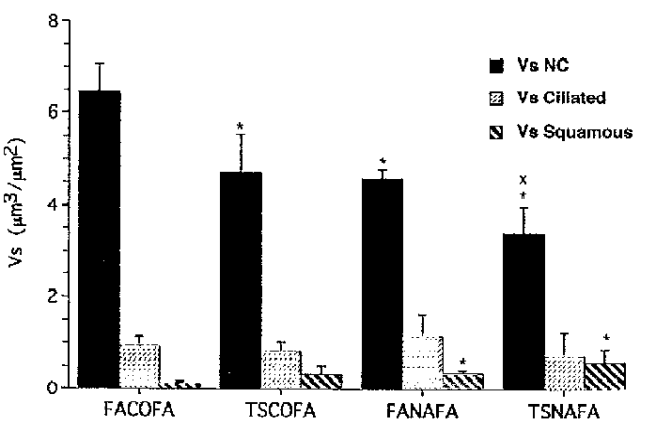

FIG. 8. The volume per surface (V_s) of the three main terminal bronchiolar epithelial cell types found in this study: nonciliated Clara cells, ciliated cells, and vacuolated cells. Note that the V_s of Clara cells (green bars) decreased for all the treatment groups compared to sham-treated controls (FACOFA). In addition, the V_s of Clara cells was significantly decreased in the group that received tobacco smoke prior to naphthalene injury (TSNAFA) compared to animals that received naphthalene alone (FANAFA). There was a significantly increased number of squamous cells in both the FANAFA and TSNAFA groups compared to the sham-treated controls (FACOFA). *Significantly different from FACOFA at $p < 0.05$; x, significantly different from FANAFA at $p < 0.05$.

TABLE 3
Volume Fraction (V_v) of Epithelial Cells in Terminal Bronchioles of Mice Exposed to ADSS or FA 14 Days after Injection with NA or CO

Group	V_v nonciliated	V_v ciliated	V_v squamous	V_v vacuolated	V_v other
FACOFA	82.41 ± 5.46	11.59 ± 1.62	1.56 ± 0.73	2.64 ± 4.98	1.80 ± 0.85
TSCOFA	71.13 ± 3.32*	12.22 ± 2.08	5.31 ± 3.73	8.00 ± 4.45	3.34 ± 1.58
FANAFA	67.61 ± 4.28*	16.65 ± 5.94	5.39 ± 1.31*	0.88 ± 1.22	9.48 ± 2.73*
TSNAFA	62.20 ± 1.86*†	12.63 ± 6.96	11.63 ± 5.74*	1.47 ± 2.47	12.07 ± 5.74*

Note. TS, smoke exposed; FA, filtered air exposed; CO, corn oil (carrier) treated; NA, naphthalene treated. All exposures/treatments are listed in chronological order to define each group. Values given are mean ± SD.

* Different from FACOFA at $p < 0.05$.

† Different from TSCOFA at $p < 0.05$.

tains 400 ± 60 $\mu g/m^3$ of naphthalene (Witschi *et al.*, 1997). The level of ADSS used in our current study (1 mg/m3) would yield a chamber concentration of approximately 1.7 $\mu g/m^3$ of naphthalene. Repeated prior treatment of mice with naphthalene has been shown to induce a state of naphthalene tolerance in the lung, whereby Clara cells become resistant to injury by even high-challenge doses (Lakritz *et al.*, 1996; O'Brien *et al.*, 1989). It is possible that even this low level of prior exposure to naphthalene present in the ETS caused a tolerizing effect and contributed to the slight resistance to injury seen in the TSNAFA group.

Initially it may seem paradoxical that prior cigarette smoke exposure produces naphthalene tolerance and protects the Clara cell from injury, but also inhibits repair and regeneration processes that occur after naphthalene injury. As the cellular mechanisms for both cytotoxicity and failure of repair remain unknown, we can only speculate as to the mechanism(s) involved. In the naphthalene injury and repair model, acute injury and exfoliation of the injured cells precedes the reparative processes of cell proliferation and redifferentiation by several days. These are distinct events. It is possible that the cell alterations that result in tolerance and the alterations that occur to produce a lack of repair are independent processes involving separate pathways. Whether alterations in the ability of a cell to withstand a toxic challenge can also subsequently result in an inability to regenerate the epithelium remains unknown. However, several possible scenarios come to mind. Our experience with naphthalene tolerance in mice would suggest that persistence of squamous cells in distal bronchioles does not occur following prior repeated exposure to naphthalene (Lakritz *et al.*, 1996). However, tobacco smoke is a complex mixture that contains many other compounds in addition to naphthalene. Prior exposure to one of the other components of smoke may be related to the lack of repair. It is also possible that the oxidant stress involved in prior smoke exposure or persistence of tobacco smoke metabolites may alter both the tolerance and repair response pathways.

The morphometric data indicate that there is a slight persistent effect of naphthalene alone in the FANAFA group at the 14-day time point. This is evidenced by slightly decreased average epithelial thickness, a decrease in V_v of nonciliated cells, and increased V_v squamated cells compared to FACOFA. However, this decrease is difficult to appreciate using standard light microscopic histopathology. It is only when a large number of airways are rigorously measured that this slight difference in the FANAFA group compared to controls (FACOFA) becomes apparent. The reason for this is amply evident when one examines the epithelium of the airway in 3-dimensions (see the terminal bronchiole of the FANAFA mice in Fig. 5C). The squamous areas are rather small and focal. In addition, when several bronchioles were examined by SEM, it was apparent that not all terminal bronchioles in the FANAFA contained patches of squamated epithelium. This underscores the importance of supplementing histopathology with rigorous morphometric measurements or 3-dimensional imaging when evaluating focal responses in pulmonary epithelium. We conclude that the bronchiolar epithelium can be considered "nearly" repaired in the FANAFA group 14 days after injury.

Very little is known about the acute pathologic changes that occur in response to concentrations of tobacco smoke that are 1 mg/m^3 or less. Previous studies have deemed this exposure level the no observed effect limit, or NOEL, for formation of ETS-related DNA adducts (Lee *et al.*, 1993). The only histopathologic changes reported at 4-fold higher doses of sidestream smoke (4 mg/m^3) are hyperplastic and metaplastic changes in the nasal turbinates of rats after 90 days (von Meyerinck *et al.*, 1989). Another study found that 5 days of exposure to 1 mg/m^3 ADSS had the sole effect of causing increased cell proliferation in the airways of a mouse strain (A/J) that is susceptible to tumors (but not in a nonsusceptible mouse strain, C57BL/6) (Rajini and Witschi, 1994). However, there was no effect of this level of ADSS exposure on general histopathology. There have been few other studies that demonstrate an acute effect of moderate levels of sidestream tobacco smoke ($\leq$ 1 mg/m^3) on deep lung respiratory epithelium in adult rodents. Our study supports this previously observed lack of histologic change; only slight epithelial alterations were observed, with a trend towards a more rounded epithelial cell

phenotype in the terminal bronchioles. However, after 16 days in filtered air, the bronchiolar epithelium of ETS-exposed mice was thinner, with a slight reduction in nonciliated cells, although this was not statistically significant. Based on the increase in the squamous cell fraction in the TSNAFA group, we conclude that the effect of smoke and naphthalene on lung repair is at least additive. This unexpected finding may indicate that there was a slight persistent tobacco smoke-related effect on Clara cells even 16 days after the last tobacco smoke exposure. The mechanism of this potential Clara cell alteration is unknown at this time, but may reflect oxidative stress on Clara cells caused by the prior smoke exposure or the downstream effect of adduct accumulation within this metabolically active cell type.

It appears that prior tobacco smoke exposure both alters the normal redifferentiation of the Clara cell population after naphthalene injury as well as results in increased persistence of squamous cells within the terminal bronchioles. Lack of Clara cell redifferentiation is indicated by diminished expression of the secretory product CC10. Our previous studies have shown that after acute injury by naphthalene in distal bronchioles, the remaining cells squamate and lack markers of the two differentiated cell types found in this region, cilia or Clara cell secretory product (Van Winkle *et al.*, 1995). Repopulation of the airway with a redifferentiated epithelium containing ciliated and Clara cells occurs over several weeks. Although there have not been previous studies that demonstrate an effect of tobacco smoke on the Clara cell redifferentiation that occurs as the epithelium regenerates in adult animals after acute injury, Ji *et al.* have shown that tobacco smoke exposure alters normal postnatal differentiation of Clara cells. During lung development in rats, ADSS exposure alters the normal Clara cell postnatal differentiation process whether the smoke exposure occurs only prenatally (Ji *et al.*, 1998) or only postnatally (Ji *et al.*, 1994). In contrast to our current findings, the effect of maternal smoke exposure alone on prenatal rat lung differentiation was increased CC10 expression (Ji *et al.*, 1998), whereas postnatal exposure had no effect on CC10 expression (Ji *et al.*, 1994). Acute Clara cell injury by bioactivated cytotoxicants during early postnatal lung development, when Clara cells are less differentiated, also results in a failure of bronchiolar repair that presents as persistent squamated epithelium in both rabbits and mice (Fanucchi *et al.*, 1997; Smiley-Jewell *et al.*, 1998). In rabbits, the unrepaired, squamated epithelium contains cuboidal cells with decreased expression of Clara cell differentiation markers including CC10 (Smiley-Jewell *et al.*, 1998).

It is possible that the lack of repair observed in our current study in mice exposed to both naphthalene and tobacco smoke may be due to the effect of naphthalene injury superimposed on undifferentiated proliferating cells already present in response to the preceding tobacco smoke exposure. Previous studies have indicated that ETS exposure enhances cell kinetic activity in terminal bronchioles in susceptible strains of mice (Rajini and Witschi 1994). Whether this is true for the strain used in our study is now under investigation. The delay in repair in our current study may also be attributed to an effect of prior ETS exposure on cell kinetic activity. Repopulation of the airway by cell proliferation both within the terminal bronchiole and at more proximal airway sites is an early step in the repair process after naphthalene injury and takes place from 1 to 4 days after naphthalene injection (Van Winkle *et al.*, 1995). Proliferation clearly occurs well before substantial redifferentiation in this model (Stripp *et al.*, 1995; Van Winkle *et al.*, 1995), so it is possible that a lack of proliferation would essentially impede epithelial redifferentiation. There is some support for this concept from studies of undifferentiated neonatal rabbit and mouse Clara cells that have decreased epithelial repair after naphthalene injury (Fanucchi *et al.*, 1997; Smiley-Jewell *et al.*, 1998). The effect of prior ETS exposure coupled with additional injury on cell kinetics remains unknown at this time and will be a subject of future research. ETS exposure also alters xenobiotic metabolizing enzymes in rat Clara cells (increasing both CYP1A1 and NADPH reductase protein expression) (Ji *et al.*, 1994). A shift in xenobiotic metabolizing or detoxifying enzymes in response to smoke exposure could also shift the intra- and extracellular targets of naphthalene metabolites to include targets required for the reparative process, thereby prolonging epithelial repair. These possibilities will be the subject of further research. To our knowledge, our study is the first to show in adult animals that a brief (5 day) prior exposure to a moderate level of ETS (1 mg/m^3) can impair repair from acute lung injury, specifically by altering normal Clara cell redifferentiation. The impact of this on lung function is unknown at this time, but secretory products of differentiated Clara cells have been shown to have a role in regulating inflammation, contribute to the mucous lining layer of the airways (Singh and Katyal, 1992), and protect cells from oxidant stress (Mango *et al.*, 1998).

Our current study indicates that prior occupational exposure to tobacco smoke may retard the ability of the lung to repair from acute injury. The lack of repair is evident as an inability to reestablish epithelial density in a timely manner in terminal bronchioles. Whether this is due to decreased epithelial proliferation or an inhibition of epithelial cell movement (either migration from proximal airways or during return to a more cuboidal shape) is unknown at this time. Further, the lack of Clara cell redifferentiation emphasizes that the factors that regulate Clara cell differentiation, both during postnatal lung development and during lung repair from injury, are still largely unknown. Acute epithelial changes in response to low levels of ETS alone have been difficult to demonstrate experimentally. This has been a puzzle to scientists trying to test the hypothesis that there is a link between low-level tobacco smoke exposure, acute lung injury, and the histologic changes that are associated with lung cancer. Our study would suggest that the effect of low-level smoke exposure is most evident as decreased potential for bronchiolar repair. We would like to

suggest that the increase in lung cancer incidence that has occurred in the latter half of the 20th century may be due to two detrimental factors present in our environment: increased exposure to inhaled or ingested environmental pollutants, which are known to directly injure the lung, increasing the incidence of acute injury, and the fact that prior exposure to tobacco smoke is also increasingly prevalent, which may result in diminished potential for epithelial repair after exposure to environmental pollutants. This research has implications for human exposures to environmental tobacco smoke that occur simultaneously with exposures to other pollutants. Future directions of this work include further definition of the nature of the acute injury/Clara cell alteration caused by prior exposure to tobacco smoke. What is not known is whether this change is permanent, whether it represents an early, preneoplastic event, or what effect this lack of Clara cell differentiation has on lung function. We conclude that tobacco smoke exposure prior to injury (1) does not change the target site or target cell type of naphthalene injury, since Clara cells in terminal bronchioles are still selectively injured, (2) may result in slightly diminished acute injury from naphthalene in distal bronchioles, and (3) delays or inhibits bronchiolar epithelial repair.

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